Genetic transformation in two potato cultivars with T-DNA from disarmed Agrobacterium

Ooms, G.; Burrell, M.M.; Karp, A.; Bevan, M.; Hille, J.

Published in:
Theoretical and Applied Genetics

DOI:
10.1007/BF00260785

Publication date:
1987

Theor Appl Genet (1987) 73:744–750

Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*

G. Ooms [1, *], M. M. Burrell [1], A. Karp [1], M. Bevan [2] and J. Hille [3, **]

[1] Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, UK
[2] Department of Molecular Genetics, Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, UK
[3] Department of Plant Molecular Biology, University of Leiden, Wassenaarseweg 66, NL-2333 AL Leiden, The Netherlands

Received September 18, 1986; Accepted November 10, 1986
Communicated by R. Riley

Summary. Derivatives of potato (*Solanum tuberosum* cv.'s 'Maris Bard' and 'Desiree') transformed with disarmed T-DNA from genetically engineered *Agrobacterium tumefaciens* strains were isolated. The transformed plants were recovered from shoot-forming tumours induced by infection of wounds with mixed-cultures of shoot-inducing *A. tumefaciens* strains T37 and either *Agrobacterium* strain LBA1834(pRAL1834), (Hille et al. 1983) or LBA4404(pBIN6; pRAL4404), (Bevan 1984). Two small-scale feasibility experiments gave at least four 'Maris Bard' plants transformed with pRAL1834 T-DNA and two 'Desiree' plants with pBIN6 T-DNA. The transformed 'Maris Bard' plants were morphologically abnormal and highly aneuploid. This was probably an unfortunate side-effect of a tissue culture-step introduced to promote the efficiency of shoot regeneration. The transformed 'Desiree' plants, in contrast, were isolated without promoting additional shoot-growth. They were morphologically normal, contained 47 and the euploid 48 chromosomes per cell respectively and had improved growth on media containing kanamycin.

Key words: Genetic manipulation – Neomycin phosphotransferase – Mixed infection – Somaclonal variation – *Solanum tuberosum*

Introduction

A range of procedures has been developed to transform plant cells with specific DNA. These include induced-uptake of DNA by protoplasts (Krens et al. 1982; Potrykus et al. 1985), fusion of protoplasts with liposomes containing DNA (Ohgawara et al. 1983; Deshayes et al. 1985) or *E. coli* spheroplasts (Hain et al. 1984), virus-mediated transformation (Brisson et al. 1984) and various adaptations of the natural transformation system of *Agrobacterium tumefaciens* (Smith and Townsend 1907). The *Agrobacterium* transformations include direct infection of wounded plant organs such as punched-out leaf discs with the bacterium (Horsch et al. 1985), co-cultivation of bacteria with cell-wall regenerating protoplasts (Marton et al. 1979), infection of rapidly growing small cell-colonies, i.e. extended co-cultivation (Muller et al. 1984; Pollock et al. 1985; An 1985; Fraley et al. 1984) and fusions of plant protoplasts with *Agrobacterium* spheroplasts (Hasezawa et al. 1981; Okado et al. 1985). In the main these transformations have been developed for tobacco or petunia. This is largely because these species are very amenable to subsequent tissue culture procedures for regenerating plants from the transformed cells. Other plant species, however, respond to only one or a few transformation regeneration procedures and some species may even require development of novel methods.

Potato is a crop of agronomical importance. It differs from tobacco and petunia in that its tissue culture is less straight forward and although plant regeneration from protoplasts or explants is possible, potato appears more susceptible to induction of morphological and cytological changes (Shepard et al. 1980; Karp and Bright 1985; Sree Ramula et al. 1983; Gunn and Day 1986), known as somaclonal variation (Larkin and Scowcroft 1981). In potato it is therefore of particular importance, that procedures for specific gene transfer combine efficiency of transformation and plant regeneration with minimal somaclonal variation. A simple and widely applicable technique would also be of advantage.

Previously we described potato cell transformation using *Agrobacterium* and the subsequent regeneration of potato

* To whom correspondence should be addressed
** Present address: Department of Molecular Biology, Agricultural University of Wageningen, De Dreyen 11, NL-6703 BC Wageningen, The Netherlands

cultivars transformed with specific DNA (Ooms et al. 1983, 1985a, b). However, the regenerated plants were transformed with shoot inducing Ti T-DNA or root inducing Ri T-DNA both of which caused specific morphological and developmental changes. More recently, following infection of wounded tissue with a single *Agrobacterium* strain, An et al. (1986) have reported the isolation of potato shoots which are kanamycin resistant. They are transformed with DNA from a binary vector plasmid but have not yet been fully characterised.

Here we report on the isolation of potato transformed with T-DNA from two types of avirulent or disarmed *Agrobacterium* strains and demonstrate the feasibility of a technically simple mixed-infection approach for the isolation of a transformed derivative of a potato cultivar which is morphologically and cytologically normal.

Materials and methods

Plant and bacterial growth

Shoot cultures of potato (*Solanum tuberosum* cvs. 'Maris Bard' and 'Desiree') were grown at 25 °C, 12 h daylength in glass jars with Petri dish lids sealed with Nesco film and then vented. The culture medium was according to Murashige and Skoog (1962), solidified with 0.9% agar and supplemented with 20 g/l sucrose but without hormones (MS20). Shoots were micro-propagated by subculturing stem/leaf segments with nodal or apical meristems, usually every three to four weeks. Transfer of tissue culture shoots into soil in pots and further culturing in a growth chamber at 12 h daylength, 18 °C day temp., 15 °C night temp. and 80–90% relative humidity gave mature plants with tubers.

The shoot inducing nopaline-type *Agrobacterium* T37 was grown on TY-medium (5 g/l tryptone, 3 g/l yeast extract pH 7.0), as was the avirulent octopine-type *Agrobacterium* strain LBA1834(pRAL1834) which contains a single Ti-plasmid inactivated for its T-DNA auxin and cytokinin gene. In contrast, the *Agrobacterium* strain LBA4404 with the plasmids pRAL4404 (Ooms et al. 1982a) and the binary plasmid pBIN6 (Bevan 1984) was grown on TY-medium with 50 mg/l kanamycin. All three strains were grown at 29 °C in liquid culture (250 rpm) or if required on plates solidified with 1.8% agar.

Transformation

Bacteria grown on plates were used to establish overnight *Agrobacterium* cultures. Mixtures were made of T37 and LBA1834 (pRAL1834) for infection of 'Maris Bard' and of T37 and LBA4404(pRAL4404; pBIN6) for infection of 'Desiree'. The bacteria were collected by centrifugation (7,000 rpm; 20 min) and used to infect wounded stems of the *in vitro* grown potato shoots. Between eight to sixteen weeks after infection, galls with spontaneous shoot formation were excised and placed on agar-solidified MS20 with 200 mg/l carbenicillin ('Maris Bard' galls) or with 200 mg/l cephotaxime (for 'Desiree' galls).

Plant regeneration

Following the initial stabilising growth period, the 'Maris Bard' tumours were transferred to MS30 (plus carbenicillin) supplemented with 2.25 mg/l BAP and 10 mg/l GA_3 to enhance the number of regenerating shoots. In contrast, 'Desiree' tumour tissues were subdivided into smaller pieces, each with one or a few shoots, and these were placed on MS20 with cephotaxime and 50 mg/l kanamycin sulfate. Shoots from 'Maris Bard' tumours were screened for octopine production (coded for by LBA1834 T-DNA) and nopaline production (T37 T-DNA coded); both assays were according to Otten and Schilperoort (1978). Of the shoots from 'Desiree' tumours only those that formed roots on media with kanamycin were tested for pBIN6 T-DNA coded nopaline production. T-DNA analysis on opine producing plants and determination of chromosome numbers in root tip squashes were done as described previously (Burrell et al. 1985; Karp et al. 1982).

Growth properties of untransformed and pBIN6 T-DNA transformed 'Desiree' were compared by placing eight comparable buds, carrying minimal leaf-stem tissue, on MS20 medium with kanamycin sulfate (Sigma K4000 lot 65F-0237 794 µg/mg) at a range of concentrations (0–400 mg/l). At each concentration, new shoot and root growth were determined by weighing the eight plantlets individually, after a culture period of three weeks, and determining the average weight and standard deviation.

Results and discussion

Transformation and plant regeneration

In a first set of experiments potato cv. 'Maris Bard' was infected with mixed bacterial cultures of oncogenic, shoot-inducing *A. tumefaciens* strain T37 and disarmed strain LBA1834(pRAL1834). Results of earlier experiments had shown that mixtures of bacterial cultures with ratios of approximately one T37 bacterium to a hundred LBA1834 (pRAL1834) bacteria did not induce significant tumour formation on potato, whereas 1:1 and 1:10 mixtures did (not shown). Since the aim of these infections was to test whether tumours induced by mixed infections could give potato plants transformed with T-DNA from the disarmed *Agrobacterium* only, the 1:10 bacterial mixtures were used. The rationale behind this choice was based on observations that tumours induced by a pure bacterial culture are mixtures. These mixtures probably contain varying proportions of untransformed cells and cells transformed with structurally different T-DNA segments resulting from independent cellular transformations (Sacristan and Melchers 1977; Ooms et al. 1982b; Van Slogteren et al. 1983). It was expected therefore, that following tumour induction by a mixture of two *Agrobacterium* cultures, the shoot promoting hormones produced by cells transformed with T37 T-DNA would stimulate not only some T37 T-DNA transformed cells to develop into shoots (characteristically without roots), but also other cells, including those transformed with non oncogenic T-DNA. This has been shown previously in tobacco (De Greve et al. 1982). Of course, regeneration of plants from cells with T-DNA's from both *Agrobacteria* used in mixed infection experiments, is possible (Ooms et al. 1982c; De Picker et al. 1986) as well as regeneration of chimeric plants (composed of different cell-types). Choosing an excess of LBA1834(pRAL1834) bacteria was expected to favour regeneration of transformed plants containing pRAL1834 T-DNA. However spontaneous development of shoots from tumours was less frequent following the mixed infections than when T37 infection was used alone and the total number of shoots recovered from a individual tumours was suppressed. To enhance this frequency, six independently induced tumours, containing one or a few shoots, were excised from the plant and cultured *in vitro* on a shoot promoting medium. This gave approximately thirty

shoots that were tested for opine biosynthesis. Of these, four contained octopine – but no nopaline biosynthesis activity (not shown).

It was noted that in the nopaline assays, potato extracts gave opine-like compounds, irrespective of whether the extracts were from leaves of untransformed potato, potato transformed with octopine-type T-DNA or from potato with nopaline type T-DNA (not shown).

From the four octopine producing shoots, four shoot culture lines were established: Mb1834A7; Mb1834B3, Mb1834B4 (the latter two from one tumour) and Mb1834E4. Subsequent analysis of these lines confirmed that the shoots contained LBA1834 T-DNA, but also that they were highly aneuploid and morphologically abnormal (see below). Since previous experiments have shown that untransformed plants from shoot forming Maris Bard tumours were essentially euploid (Ooms et al. 1983), it appeared likely that the present aneuploidy was at least partly caused by the additional *in vitro* culture step. Therefore, in a second series of experiments this step was avoided.

Shoot cultures of cv. 'Desiree' were infected with mixed bacterial cultures of T37 and LBA4404(pRAL4404;pBIN6) and plants were regenerated from shoot forming tumours. Cultivar 'Desiree' was chosen because spontaneous shoot formation from T37 tumours was generally more frequent in this cultivar and transformation with pBIN6 T-DNA enabled selection for growth on kanamycin containing media prior to opine identification. From six 'Desiree' tumours with shoots, a total of five shoots were selected that spontaneously formed roots on a medium with kanamycin and of these, only two produced nopaline and upon retesting consistently formed roots on kanamycin-containing medium. The lines established from these shoots were DB6C6 and DB6H4.

Transformed plants

Lines established from the four putatively transformed 'Maris Bard' shoots and the two selected 'Desiree' shoots were examined in detail for growth properties and analysed to obtain further proof of transformation.

Micropropagation of all six lines did not reveal significant changes in morphology which suggested that they were genetically stable. However, considerable differences were evident in morphology between the lines. 'Maris Bard' derived line Mb1834A7 did not form roots. Mb1834E4 had many leaves per shoot, as did Mb1834B4, but in the latter case the leaves were positioned slightly different and the axillary buds often lacked dormancy (Fig. 1). Mb1834B3 (from the same initial tumour as Mb1834B4) had a dwarfed phenotype and spontaneously formed (sprouting) tubers from its axillary buds (Fig. 1). This observation is consistent with a pleiotropic effect of diminished gibberellin activity on stem elongation and on inhibition of tuber formation (Slater 1963; Lovell and Booth 1967; Tizio 1972; Kumar and Wareing 1974). 'Desiree' derived lines DB6C6 and DB6H4 were morphologically indistinguishable from each other and from 'Desiree', irrespective of whether they were compared as shoot cultures *in vitro*, as plants in soil or as tubers.

The morphological variation between 'Maris Bard'-derived shoots and the uniformity among 'Desiree'-

Fig. 1. Single shoots from cv. 'Maris Bard' and potato lines transformed with T-DNA from LBA1834(pRAL1834). From left to right cv. 'Maris Bard', Mb1834A7, Mb1834E4, Mb1834B4 and Mb1834B3

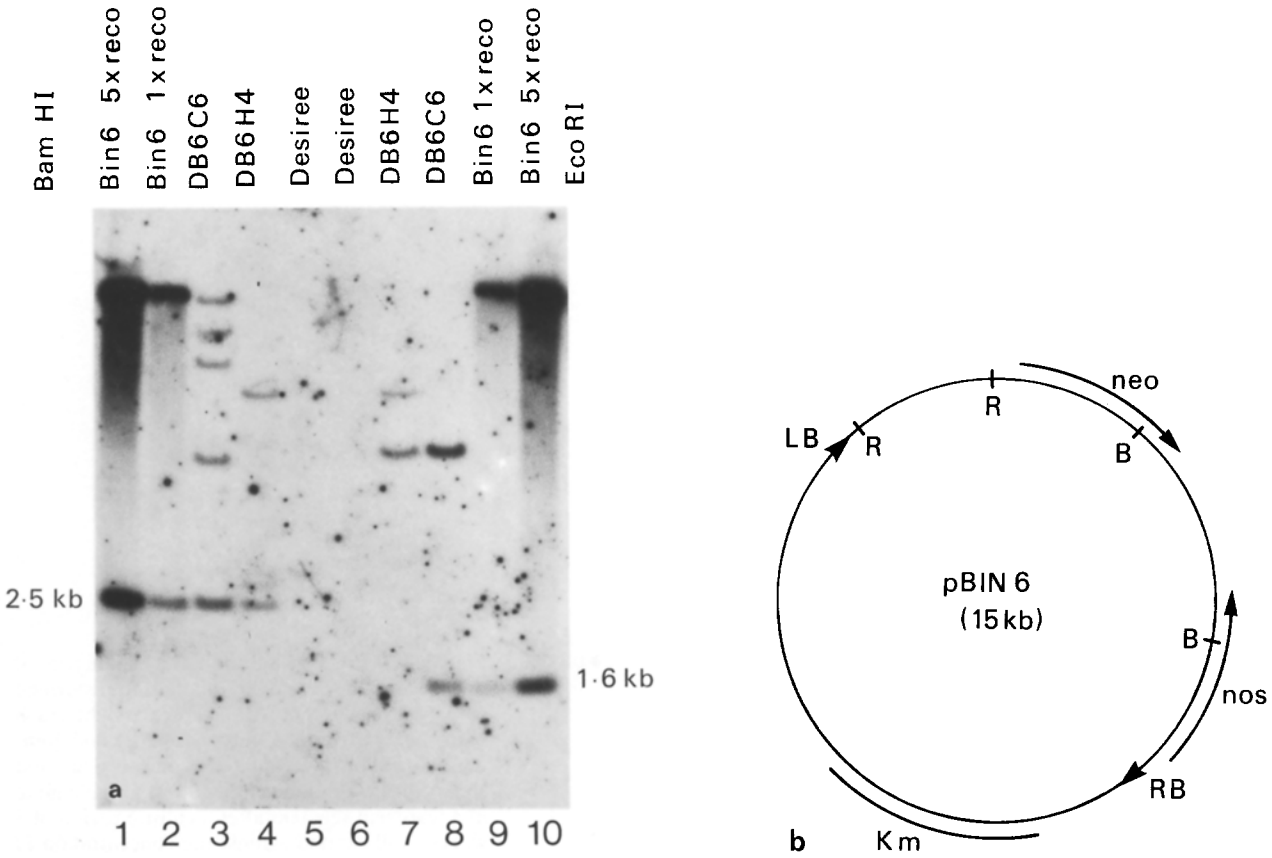

Fig. 2. Hybridization patterns of DNA from potato transformed with pBIN6 T-DNA (**a**) and functional and structural map of plasmid pBIN6 (**b**). **a** An autroradiogram illustrating the T-DNA banding pattern obtained after exposure of a Southern blot with DNA from untransformed 'Desiree' (lanes 5, 6), from transformed lines DB6C6 (lanes, 3, 8) and DB6H4 (lanes 4, 7) and with pBIN6 DNA at amounts equivalent to one or to five pBIN6 molecules per tetraploid potato genome (lanes 2, 9 and lanes 1, 10, respectively). The DNA was treated with restriction endonuclease BamHI (lanes 1–5) and EcoRI (lanes 6–10) and hybridised with ^{32}P-labelled pBIN6 DNA. **b** Circular map of plasmid pBIN6 with the relative positioning of EcoRI sites (R); BamHI sites (B); left and right T-DNA border sequences (LB and RB); bacterial- and plant neomycin phosphotransferase genes (Km and neo, respectively) and the gene for nopaline synthase (nos)

derived shoots was correlated with the extent of variation found in chromosome numbers. Mb1834E4 had ninety-three chromosomes per cell, Mb1834B4 ninety-two and Mb1834B3 seventy-three. Since Mb1834A7 made no roots, its chromosome number was not determined. Line DB6C6 had the euploid number of 48 chromosomes per cell and DB6H4 was (slightly) aneuploid with 47 chromosomes per cell. Although these results indicated that the morphological variation between the 'Maris Bard' derived shoots can largely be explained by chromosomal variation, some influence of the remaining non-mutated T-DNA genes from LBA1834 (Hille et al. 1983), probably co-introduced with the octopine synthase marker cannot be excluded.

Molecular proof that the selected lines were transformed was obtained from DNA hybridization-analysis (Southern 1976) of blots prepared with DNA isolated from Mb1834A7, Mb1834B3, DB6C6 and DB6H4. The DNA was digested with restriction endonucleases EcoRI and BamHI. After restriction fragment separation in 0.7% agarose and Southern blotting onto a membrane filter, the DNA from the 'Maris Bard' transformants was hybridised with ^{32}P labelled plasmid pRAL3076, a recombinant DNA plasmid (Ooms et al. 1981) with substantial DNA homology with the T-DNA region of plasmid pRAL1834 (Hille et al. 1983). The DNA from the 'Desiree' transformants was hybridised with ^{32}P-labelled DNA of the plasmid pBIN6 (Bevan 1984).

The pRAL3076 hybridizations showed that at least some LBA1834 derived T-DNA sequences, not detected in 'Maris Bard' DNA, were present in approximately one or two copies per tetraploid genome (not shown). The pBIN6 hybridization showed homologous sequences detected in DB6C6 and DB6H4 DNA but not in 'Desiree' DNA, (Fig. 2 a). DB6C6 had approximately two or three T-DNA copies and DB6H4 perhaps only one. This was concluded from the relative intensities of

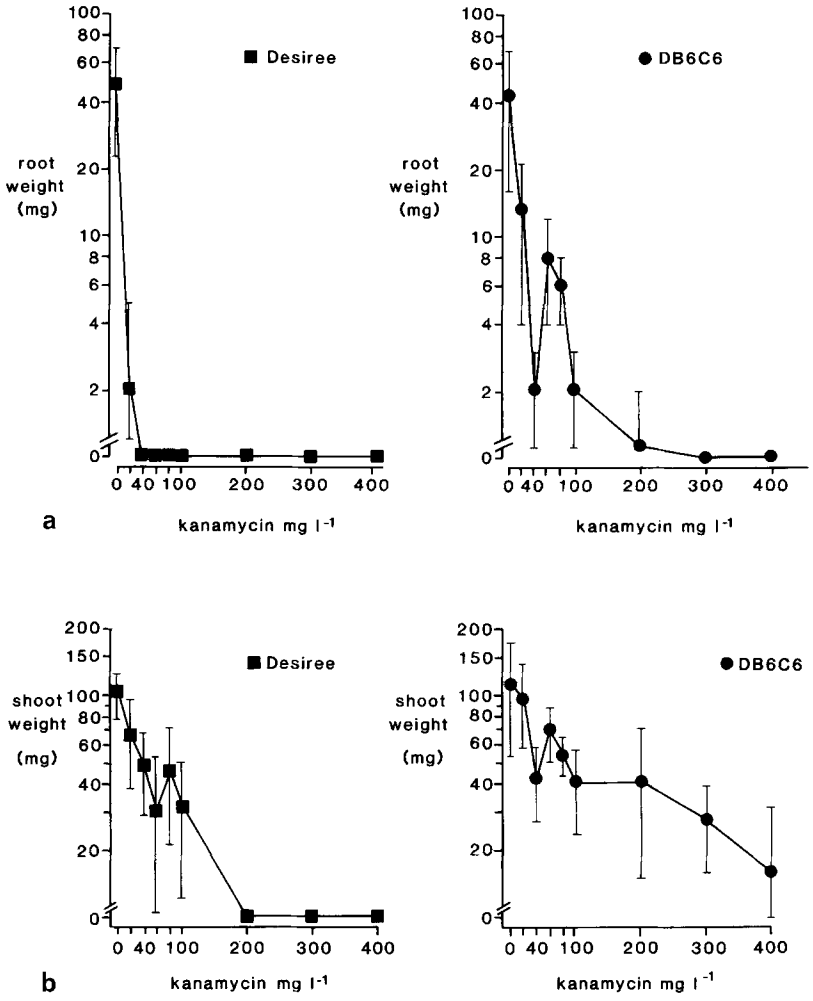

Fig. 3a, b. Growth inhibition by kanamycin of untransformed potato and potato transformed with pBIN6 T-DNA. The average weight (each point is taken from 8 measurements) and standard deviation (vertical bars) of roots (**a**) and shoots (**b**) that had developed from a single node in a leaf-stem segment after a culture period of 3 weeks, is illustrated against the concentration of kanamycin in the growth medium (left-untransformed potato cv. 'Desiree'; right-transformed line DB6 C6)

bands at the position of an internal 2.5 kb BamHI fragment (Fig. 2b), representing hybridization to 5× and 1× reconstruction DNA (Fig. 2a; lanes 1 and 2) and to DB6C6 and DB6H4 DNA (Fig. 2a, lanes 3 and 4). The detection of four bands in lane 3 and only two bands in lane 4, none of which corresponded with hybridization to internal pBIN6 T-DNA fragments, supported the difference in estimated copy number. These latter bands may represent fragments containing some pBIN6 T-DNA and plant DNA or junction fragments in which DNA from multiple T-DNA segments are joined together. It is noted that DB6C6 also contained an internal 1.6 kb EcoRI fragment (Fig. 2a, lane 8), which like the 2.5 kb BamHI fragment, was located entirely within the pBIN6 T-DNA region (Fig. 2b). This 1.6 kb EcoRI fragment was not detected in DB6H4 DNA. (Fig. 2a, lane 7) suggesting that DB6H4 contained a truncated T-DNA copy not flanked by both of the pBIN6 left and right border (LB/RB) sequences (Fig. 2b). It is further noted that the relatively low copy number of pBIN6 T-DNA in both the transformed 'Desiree' lines contrasts with the higher copy number of approximately 5–20, found in four tobacco lines obtained after transformation with the same *Agrobacterium* strain using a slightly different procedure (Bevan 1984).

Kanamycin resistance

The reduced sensitivity of DB6C6 and DB6H4 to kanamycin, conferred by neomycin phosphotransferase, coded for by the pBIN6 T-DNA, was determined by comparing growth of Desiree and the transformed lines on a series of media with differing concentrations of kanamycin. The results for DB6C6 and Desiree are illustrated in Fig. 3; essentially identical results were obtained for DB6H4 (not shown). It was concluded that root growth from axillary buds of Desiree was already inhibited at 40 mg/l kanamycin. In contrast, axillary buds of transformed 'Desiree' showed occasional root growth even at 200 mg/l kanamycin. Complete inhibition of shoot growth required higher concentrations

of kanamycin of up to 200 mg/l in the case of untransformed 'Desiree'. This concentration impeded growth of transformed 'Desiree', but did not prevent it. Figure 3 also illustrates that considerable variation was evident in growth between individual shoots. Although root growth was prevented from the initial axillary buds, occasionally axillary buds from newly formed shoots produced (aerial) roots that could grow into the kanamycin-containing medium.

Based on the growth inhibition curves for root and shoot formation other selection schemes could also be designed for future isolation of potato transformed with pBIN6 nos-neo type selectable markers. Equally, the curves will be of use in comparing efficiency of selection based on acquired reduced-sensitivity to kanamycin with other possible selection criteria such as reduced sensitivity to hygromycin or the effect of different regulatory sequences on expression of the selectable marker genes (Waldron et al. 1985).

Conclusion

In conclusion, the principle has been demonstrated that derivatives of potato cultivars transformed with non-oncogenic T-DNA can be isolated. In this particular case we used mixed infections as an approach, both with and without selection and with and without concurrent acquisition of significant chromosomal variation. Analysis of the reduction in sensitivity to kanamycin of transformed plants is of potential use in the design of alternative selection schemes based on pBIN6-type transformations and for comparison with alternative selection methods.

Acknowledgements. Assistance in various parts of this work by J. Roberts, R. Risiott, M. Bossen and J. Atkinson is acknowledged. This work was carried out under MAFF licence PHF26A issued under the Plant Pests (Great Britain) Order 1980.

References

An G (1985) High efficiency transformation of cultured tobacco cells. Plant Physiol 79:568–570

An G, Watson BD, Chiang CC (1986) Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using a binary Ti vector system. Plant Physiol 81:301–305

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acid Res 12:8711–8721

Brisson N, Paszkowski J, Penswick JR, Gronenborn B, Potrykus I, Hohn T (1984) Expression of a bacterial gene in plants using a viral vector. Nature 310:511–514

Burrell MM, Twell D, Karp A, Ooms G (1985) Expression of Ti T-DNA in differentiated tissues of potato (*Solanum tuberosum* cv. 'Maris Bard'). Plant Mol Biol 5:213–222

De Greve H, Leemans J, Hernalsteens JP, Thia-Toong L, De Beuckeleer M, Willmitzer L, Otten L, Van Montagu M, Schell J (1982) Regeneration of normal and fertile plants that express octopine synthase, from tobacco crown galls after deletion of tumour-controlling functions. Nature 300: 752–755

De Picker A, Herman L, Jacobs A, Schell J, Van Montagu M (1986) Frequencies of simultaneous transformation with different T-DNA and their relevance to the *Agrobacterium* and plant cell interaction. Mol Gen Genet 201:477–484

Deshayes A, Herrera-Estrella L, Caboche M (1985) Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. EMBO J 4:2731–2739

Fraley, RT, Horsch RB, Matzke A, Chilton MD, Chilton WS, Sanders PR (1984) *In vitro* transformation of petunia cells by an improved method of cocultivation with *Agrobacterium tumefaciens* strains. Plant Mol Biol 3:371–378

Gunn RE, Day PR (1986) *In vitro* culture in plant breeding In: Withers LA, Alderson PG (eds) Plant tissue culture and its agricultural applications. Butterworths, London, pp 313–336

Hain R, Steinbiss HH, Schell J (1984) Fusion of *Agrobacterium* and *E. coli* spheroplasts with *Nicotiana tabacum* protoplasts: Direct gene transfer from micro-organism to higher plant. Plant Cell Rep 3:60–64

Hasezawa S, Nagata T, Syono K (1981) Transformation of *Vinca* protoplasts mediated by *Agrobacterium* spheroplasts. Mol Gen Genet 182:206–210

Horsch RB, Fry SE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 277:1229–1231

Hille J, Wullems GJ, Schilperoort RA (1983) Non-oncogenic T-region mutants of *Agrobacterium tumefaciens* do transfer T-DNA into plant cells. Plant Mol Biol 2:155–163

Karp A, Nelson RS, Thomas E, Bright SWJ (1982) Chromosome variation in protoplast-derived potato plants. Theor Appl Genet 63:265–272

Karp A, Bright SWJ (1985) On the causes and origins of somaclonal variation. In: Miflin BJ (ed) Oxford surveys of plant molecular and cell biology. Oxford University Press, London, pp 199–325

Krens FA, Molendijk L, Wullems GJ, Schilperoort RA (1982) *In vitro* transformation of plant protoplasts with Ti plasmid DNA. Nature 296:72–74

Kumar D, Wareing PF (1974) Studies on tuberization of *Solanum andigena* II growth hormones and tuberization. New Phytol 73:833–840

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60:197–214

Lovell PH, Booth A (1967) Effects of gibberellic acid on growth, tuber formation and carbohydrate distribution in *Solanum tuberosum*. New Phytol 66:525–537

Marton L, Wullems GJ, Molendijk L, Schilperoort RA (1979) In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. Nature 277:129–131

Muller A, Manzara T, Lurquin PF (1984) Crown gall transformation of tobacco callus by co-cultivation with *Agrobacterium tumefaciens*. Biochem Biophys Res Commun 123:458–462

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Ohgaware T, Uchimiya H, Harada H (1983) Uptake of liposome-encapsulating plasmid DNA by plant protoplasts and molecular fate of foreign DNA. Protoplasma 116: 145–148

Okado K, Hasazawa S, Syono K, Nagata T (1985) Further evidence for the transformation of vinca rosea protoplasts by *Agrobacterium tumefaciens* spheroplasts. Plant Cell Rep 4:133–136

Ooms G, Hooykaas PJJ, Moolenaar G, Schilperoort RA (1981) Crown gall plant tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmids: analysis of T-DNA functions. Gene 14:33–50

Ooms G, Hooykaas PJJ, Van Veen RJM, Van Beelen P, Regensburg TJG, Schilperoort RA (1982a) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid 7:15–29

Ooms, G. Bakker A, Molendijk L, Wullems GJ, Gordon MP, Nester EW, Schilperoort RA (1982b) T-DNA organization in homogeneous and heterogeneous octopine type crown gall tissues of *Nicotiana tabacum*. Cell 30:589–597

Ooms G, Molendijk L, Schilperoort RA (1982c) Double infection of tobacco plants by two complementing octopine T-region mutants of *Agrobacterium tumefaciens*. Plant Mol Biol 1:217–227

Ooms G, Karp A, Roberts J (1983) From tumour to tuber: tumour cell characteristics and chromosome numbers of crown gall-derived tetraploid potato plants (*Solanum tuberosum* cv. 'Maris Bard'). Theor Appl Genet 66:169–172

Ooms G, Karp A, Burrell MM, Twell D, Roberts J (1985) Genetic modification of potato development using Ri T-DNA. Theor Appl Genet 70:440–446

Otten LABM, Schilperoort RA (1978) A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim Biophys Acta 527:494–500

Pollock K, Barfield DG, Robinson SJ, Shields R (1985) Transformation of protoplast-derived cell colonies and suspension cultures by *Agrobacterium*-tumefaciens. Plant Cell Rep 4:202–205

Potrykus I, Shillito RD, Saul MW, Paszkowski J (1985) Direct gene transfer: state of the art and future potential. Plant Mol Rep 3:117–128

Sacristan MD, Melchers G (1977) Regeneration of plants from habituated and *Agrobacterium*-transformed single clones of tobacco. Mol Gen Genet 152:111–117

Shepard JF, Bidney D, Shahin E (1980) Potato protoplasts in crop improvment. Science 208:17–24

Slater JW (1963) Mechanisms of tuber initiation. In: Ivins JD, Milthorpe FL (eds) The growth of the potato. Butterworth, London, pp 114–120

Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. Science 25:671–673

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Sree Ramulu K, Dijkhuis P, Roest S (1983) Phenotypic variation and ploidy level of plants regenerated from protoplasts of tetraploid potato (*Solanum tuberosum* L cv. 'Bintje'). Theor Appl Genet 65:329–338

Tizio R (1972) Effet de la lumière sur la tubérisation de la pomme de terre. Potato Res 15:257–262

Van Slogteren GMS, Hoge JHC, Hooykaas PJJ, Schilperoort RA (1983) Clonal analysis of heterogeneous crown gall tissues induced by wild-type and shooter mutant strains of *Agrobacterium* tumefaciens expression of T-DNA genes. Plant Mol Biol 2:321–335

Waldron C, Murphy EB, Roberts JL, Gustafson GD, Armour SL, Malcolm SK (1985) Resistance to hygromycin B. Plant Mol Biol 5:103–108